

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Paula M. Olofso et al. Examiner: Stuart F Baum
Serial No.: 10/813,482 Group Art Unit: 1638
Filed: March 30, 2004 Docket No.: 600.479US2
Title: METHOD TO ENHANCE AGROBACTERIUM-MEDIATED
TRANSFORMATION OF PLANTS

RULE 132 DECLARATION

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Dr. Todd Jones, declare and say as follows:

1. I received a Bachelor of Science in Botany from the University of California, Davis in 1983, a Master of Science in Botany from the University of California, Davis in 1984, and a Doctor of Philosophy in Plant Cell and Developmental Biology from the University of California, Davis in 1988. From 1988-1989, I was a post-doctoral fellow in the Department of Genetics at the University of California, Berkeley. From 1989-2001, I was a Research Biologist at DuPont Crop Genetics, and from 2001-2004, I was Director of Forest Biotechnology at Weyerhaeuser Company. Since 2004, I have been a Technology Center Leader at the Cell Biology Technology Center at BASF Plant Science. BASF Plant Science GmbH is one of the co-assignees of the present application. I am a member of numerous professional societies, a co-inventor named on three patent applications, and a co-author of eighteen journal articles. I make this Declaration in support of the patentability of the claims in the above-identified application.

2. In the Office Action dated January 5, 2009 for the above-referenced matter, the Examiner rejected claims 57-60, 62-67, 71-73, and 75-78 under 35 U.S.C. § 103(a) as being unpatentable over Enriquez-Obregón et al. (Biotechnologia Aplicada, 14:169 (1997)) in view of Hansen et al. (Trends in Plant Science, 4:226 (1999)).

3. Enriquez-Obregón et al. report on the effect of a combination of ascorbic acid, cysteine and silver nitrate in pre-coculture liquid medium, co-culture medium, or both, on

Agrobacterium-mediated transformation in sugarcane (Table 3). Cysteine was employed individually at 40 mg/L or 90 mg/L in experiments to determine percent explant viability (Table 2) or in the combination at 40 mg/L to determine percent explant viability (Table 2) or to determine percent GUS positive explants, percent herbicide resistance or the presence of a transferred gene after *Agrobacterium*-mediated transformation (Table 3). When cysteine was tested individually at 40 mg/L for viability after *Agrobacterium* infection, the explant was embryogenic while at 90 mg/L the explant was nonembryogenic (Table 2).

4. In the absence of data, there is no reason to expect that a combination of agents, let alone an individual agent, at any particular concentration would enhance stable transformation. As discussed below, combinations of agents may in fact result in lower transformation efficiencies than one of the individual agents.

5. Experiments were conducted by individuals in the employ of BASF where cysteine was present in co-cultivation medium. The experiments included those that used embryogenic *Brachypodium distachyon*, wheat and maize cultures infected with *Agrobacterium*.

6. For the *Brachypodium distachyon* (a monocot grass) experiments, calli from seed from two different lines were cultured on media with 100 mg/L cysteine. Although no enhancement of transformation was observed with cysteine treatment, the experiments were not intended to test the effect of cysteine on transformation. However, the concentration of cysteine employed in this experiment was similar to one of the concentrations which enhanced viability in Enriquez-Obregón et al. and was over two times greater than the concentration of cysteine employed in the combination of agents which was used in transformation experiments in Enriquez-Obregón et al.

7. Various concentrations of cysteine were incorporated into co-cultivation media on which *Agrobacterium*-infected embryogenic wheat cultures of Canon and Bobwhite 56 were placed. The data for Canon wheat transformation is attached hereto (Figure 1). It is noteworthy that concentrations of cysteine of 300 mg/L and 350 mg/L were most effective at enhancing

transformation, whereas lower and higher concentrations did not result in a significantly different effect or resulted in an inhibitory effect relative to control cultures.

8. Cysteine concentrations of 150 and/or 300 mg/L were tested for enhancement of transformation efficiencies of immature embryos from two different lines of corn (inbred J553 and hybrid J553X(HxA)). Enhanced transformation efficiencies were observed at both cysteine concentrations (Tables 1-2).

9. Combinations of cysteine and glutathione, ascorbic acid or silver nitrate were also tested for enhancement of corn transformation efficiencies (Tables 3-7). Combinations of 300 mg/L cysteine with 100 mg/L ascorbic acid, and 150 mg/L cysteine and 153 mg/L glutathione, decreased inbred corn transformation efficiency relative to embryos treated with the individual agents (Tables 3-4). Although a combination of 150 mg/L cysteine and 153 mg/L glutathione resulted in a slightly higher transformation efficiency in the hybrid line, those results may not be statistically significant (Table 5). A decrease in hybrid transformation efficiency was observed when a cysteine concentration of 300 mg/L was employed with a silver nitrate concentration of 1.27 mg/L relative to a cysteine concentration of 150 mg/L and a silver nitrate concentration of 1.27 mg/L (Table 7). A decrease in inbred transformation efficiency was observed when a cysteine concentration of 300 mg/L was employed with 1.27 mg/L silver nitrate or with 100 mg/L ascorbic acid relative to 300 mg/L cysteine alone (Table 7).

10. Thus, transformation efficiency in corn was most often decreased when a combination of cysteine and ascorbic acid, glutathione or silver nitrate was employed.

11. In view of the results discussed above, as well as the results for soybean in the above-referenced application, the use of a combination of agents by Enriquez-Obregón et al. may have actually decreased transformation efficiency. Such a conclusion may be supported by the viability data in Table 2 in Enriquez-Obregón et al., as the explant viability observed with each of the individual agents was not additive when the agents were employed in combination. In addition, the concentration of cysteine employed by Enriquez-Obregón et al. in transformation

experiments may not have had any, or only a marginal, effect on transformation, as concentrations of cysteine of less than 200 mg/L or 300 mg/L did not enhance wheat or soybean, respectively, transformation efficiencies.

12. The position of the Examiner, that one of skill in the art would be motivated to test higher concentrations of cysteine in view of Enriquez-Obregón et al., is contrary to the teachings in Enriquez-Obregón et al. First, Enriquez-Obregón et al. clearly teach the use of a combination of agents for "improved" transformation efficiency and regeneration of embryogenic cultures. Second, for those transformation experiments, Enriquez-Obregón et al. selected the lower concentration of the two tested concentrations of each of the three agents to include in pre-coculture liquid medium and co-culture medium (Table 3).

13. Further, "higher" concentrations of cysteine do not necessarily result in enhanced transformation. For instance, concentrations of cysteine of 400 mg/L or more did not enhance wheat transformation (see attached Figure 1) and concentrations of cysteine of 1000-2000 mg/L resulted in decreased soybean transformation efficiencies relative to lower cysteine concentrations (see Figure 9 in the above-referenced specification). And for *Brachypodium distachyon*, cysteine concentrations over twice those used in Enriquez-Obregón et al. did not enhance transformation.

14. Therefore, it would not be obvious to one of skill in the art in view of Enriquez-Obregón et al. to employ cysteine or other sulfhydryl-containing agents alone in co-cultivation media to enhance plant transformation of embryogenic cultures. Moreover, it was unexpected that concentrations of cysteine that were much higher than those employed in Enriquez-Obregón et al. would yield enhanced transformation efficiencies in plants such as soybean, wheat and corn.

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15. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 1 Apr. 1 2009

By: Todd Jones

Dr. Todd Jones

Figure 1

Effect of L-Cysteine

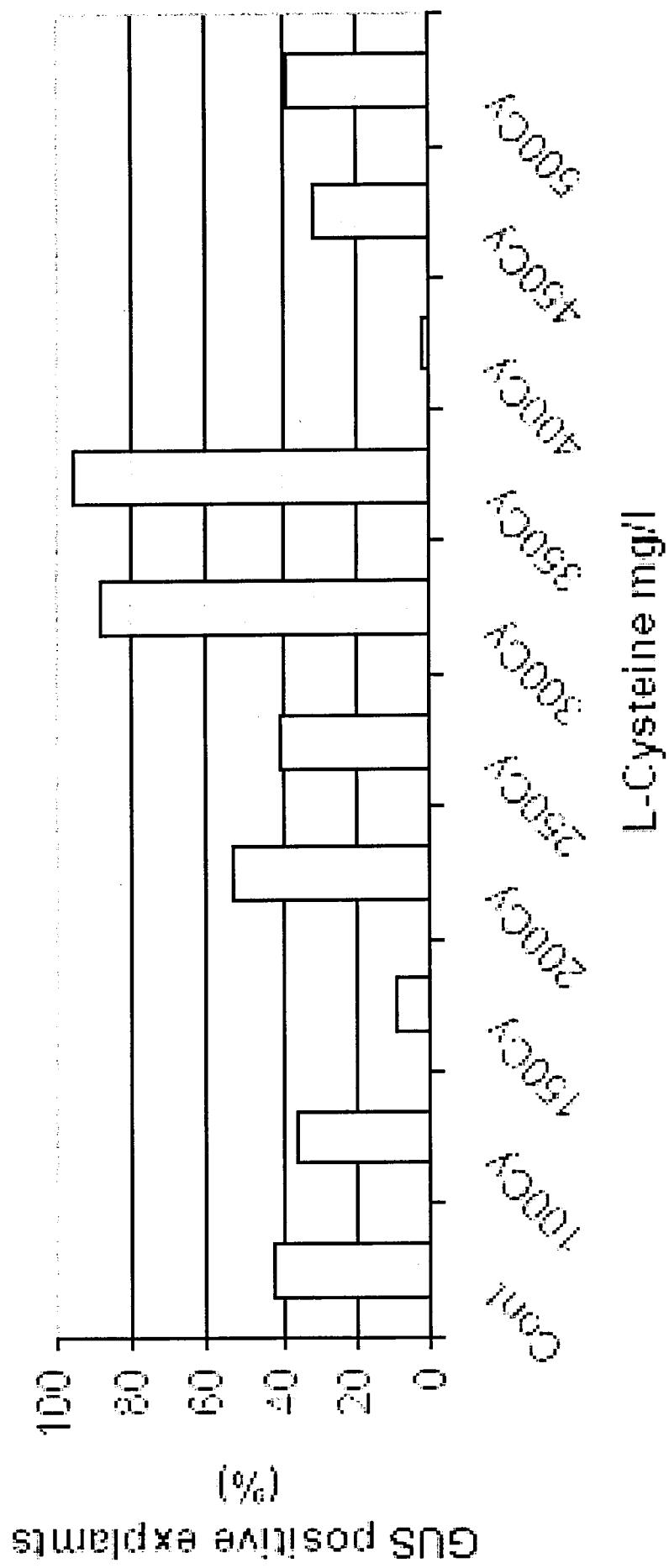


Table 1. Effect of L-cysteine on corn transformation. Transformation experiments were conducted with the mutated *ahas* gene as the selection marker.

Genotype	L-cysteine (2.48 mM)	# Rep	AVG TE%	STDEV
J553X(HxA)	0	4	9.5	7.1
	2.48	4	33.1	18.5
J553	0	6	1.2	1.4
	2.48	6	9.8	5.8

Table 2. Effect of L-cysteine on J553x(HIIAxA188) hybrid line transformation. Transformation experiments were conducted with the mutant *ahas* gene as the selection marker.

L-Cysteine (mM)	Number of replicates	Embryo Amount	Num Conf Events	Confirmed Efficiency	STDEV
0	4	179	17	9.5	7.1
1.24	4	164	30	19.0	7.9
2.48	4	195	35	17.9	6.3

Table 3. Effect of L-cysteine (L-Cys) and glutathione (L-GT) in co-cultivation media on J553 inbred line corn transformation. Transformation constructs contained the mutated *ahas* gene as the selection marker.

L-cysteine (mM)	L-GT (mM)	Rep	# IEs	# Events	TE %	STDEV
0	0	5	262	4	1.9	3.2
1.24	0	5	260	42	15.2	12.8
0	0.5	5	234	33	14.9	22.7
1.24	0.5	5	230	9	4.5	10.1

Table 4. Effect of L-cysteine (L-Cys) and glutathione (L-GT) in co-cultivation media on J553 inbred line corn transformation. Transformation constructs contained the *dsdA* gene as the selection marker.

L-cysteine (mM)	L-GT (mM)	Rep	# IEs	# Events	TE %	STDEV
0	0	3	136	5	3.3	3.6
1.24	0	3	131	24	21.3	19.9
0	0.5	3	121	18	16.2	7.7
1.24	0.5	3	129	6	4.8	5.9

Table 5. Effect of L-cysteine (L-Cys) and glutathione (L-GT) in co-cultivation media on J553x(HIIAxA188) hybrid corn transformation. Transformation constructs contained the *dsdA* gene as the selection marker.

L-cysteine (mM)	L-GT (mM)	Rep	# IEs	# Events	TE %	STDEV
0	0	5	260	18	5.7	6.4
1.24	0	5	269	57	18.6	12.9
0	0.5	5	282	56	16.0	16.1
1.24	0.5	5	241	58	22.3	5.7

Table 6. Effect of L-cysteine, ascorbic acid, and AgNO₃ in co-cultivation media on J553x(HiIIAxA188) hybrid line transformation. Transformation experiments were conducted with the mutated *ahas* gene as the selection marker.

L-cysteine (mM)	Ascorbic acid (mM)	AgNO ₃ (uM)	Embryo Amount	Num Conf Events	Confirmed Efficiency	STDEV
0	0.57	0	191	20	10.6	19.9
1.24	0	7.5	108	26	22.6	29.1
2.48	0	0	189	44	22.3	13.5
2.48	0	7.5	188	28	14.6	6.6
2.48	0.57	0	199	0	0.0	0.0

Table 7. Effect of L-cysteine, ascorbic acid, and AgNO₃ in co-cultivation media on J553 inbred line transformation. Transformation experiments were conducted with the mutated *ahas* gene as the selection marker.

L-cysteine (mM)	Ascorbic acid (mM)	AgNO ₃ (uM)	Embryo Amount	Num Conf Events	Confirmed Efficiency	STDEV
2.48	0	0	202	27	12.6	9.7
2.48	0	7.5	203	11	5.2	2.1
0	0.57	0	183	0	0	0
2.48	0.57	0	178	0	0	0